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# Opposite Effects of $Mn^{2+}$ and $Zn^{2+}$ on PsaR-Mediated Expression of the Virulence Genes *pcpA*, *prtA*, and *psaBCA* of *Streptococcus pneumoniae*<sup>▽</sup>

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Homeostasis of  $Zn^{2+}$  and  $Mn^{2+}$  is important for the physiology and virulence of the human pathogen *Streptococcus pneumoniae*. Here, transcriptome analysis was used to determine the response of *S. pneumoniae* D39 to a high concentration of  $Zn^{2+}$ . Interestingly, virulence genes encoding the choline binding protein PcpA, the extracellular serine protease PrtA, and the  $Mn^{2+}$  uptake system PsaBC(A) were strongly upregulated in the presence of  $Zn^{2+}$ . Using random mutagenesis, a previously described  $Mn^{2+}$ -responsive transcriptional repressor, PsaR, was found to mediate the observed  $Zn^{2+}$ -dependent derepression. In addition, PsaR is also responsible for the  $Mn^{2+}$ -dependent repression of these genes. Subsequently, we investigated how these opposite effects are mediated by the same regulator. In vitro binding of purified PsaR to the *prtA*, *pcpA*, and *psaB* promoters was stimulated by  $Mn^{2+}$ , whereas  $Zn^{2+}$  destroyed the interaction of PsaR with its target promoters. Mutational analysis of the *pcpA* promoter demonstrated the presence of a PsaR operator that mediates the transcriptional effects. In conclusion, PsaR is responsible for the counteracting effects of  $Mn^{2+}$  and  $Zn^{2+}$  on the expression of several virulence genes in *S. pneumoniae*, suggesting that the ratio of these metal ions exerts an important influence on pneumococcal pathogenesis.

The human pathogen *Streptococcus pneumoniae* is a commensal of the nasopharynx, but it can become virulent and infect the lungs, middle ear, brain, and bloodstream, causing severe diseases such as pneumonia, otitis media, meningitis, or sepsis (see references 10 and 61 for reviews). Little is known about the specific environmental changes that *S. pneumoniae* encounters during infection of the human body and the way in which these affect virulence. One important environmental factor is probably the concentration of metal ions, which form a class of nutrients that are important for bacteria in small amounts but are often toxic in larger amounts (9, 20, 57, 58).

An important metal ion that *S. pneumoniae* could face is  $Zn^{2+}$ , which is present in the human body in concentrations ranging from a few  $\mu M$  to over 100  $\mu M$  (81). In the host,  $Zn^{2+}$  is of great importance for immunity, as it is necessary for proper functioning of immune cells (28), and mild  $Zn^{2+}$  deficiency severely affects immune function (72).  $Zn^{2+}$  levels are elevated during inflammation (53), and  $Zn^{2+}$  administration reduces airway infections in children in developing countries (71). Moreover,  $Zn^{2+}$  deficiency results in an increased risk of pneumococcal infection and death in mice (75) and in a lower immune response to the pneumococcal antigen PspA (76). Interestingly, several studies suggest that the use of and response to metal ions, such as  $Zn^{2+}$ ,  $Mn^{2+}$ ,

and  $Fe^{2+}$ , is important for the virulence and physiology of pathogenic streptococci (3, 11, 12, 18, 30, 32, 39, 40, 46, 50, 54, 55, 63, 64, 66, 67).

In *S. pneumoniae* several systems dedicated to the acquisition of specific metal ions have been studied. These are the PsaBCA  $Mn^{2+}$  transporter (18, 44, 50), the AdcCBA  $Zn^{2+}$  uptake system (18, 19), and three iron uptake loci (*piaABCD*, *piuBCDA*, and *pit*) (11, 12). The PsaBCA complex is involved in virulence, oxidative stress, penicillin stress, competence, and adhesion via interaction with human E-cadherin (2, 6, 18, 32, 48, 50, 59, 60, 79). Expression of the *psaBCA* genes is repressed by the DtxR family regulator PsaR in response to high  $Mn^{2+}$  concentrations (31). *S. pneumoniae* also contains systems involved in cation efflux (58), such as *czcD*, which has recently been shown to be responsible for  $Zn^{2+}$  resistance (39). The presence in *S. pneumoniae* of this  $Zn^{2+}$  efflux system together with the AdcCBA  $Zn^{2+}$  uptake system indicates that this pathogen has to deal with fluctuating  $Zn^{2+}$  levels in the human body.

Therefore, we explored the response of *S. pneumoniae* to a high  $Zn^{2+}$  concentration by means of transcriptome analysis. Interestingly,  $Zn^{2+}$  was found to increase expression of several genes involved in virulence, namely, *prtA*, *pcpA*, and *psaBC*, which could be counteracted by  $Mn^{2+}$ . Subsequent research showed that the transcriptional regulator PsaR is directly responsible for the  $Mn^{2+}$ -dependent repression and the  $Zn^{2+}$ -dependent derepression of these genes.

## MATERIALS AND METHODS

**DNA techniques,  $\beta$ -galactosidase assays, bacterial strains, and growth conditions.** All DNA manipulation techniques, growth conditions, and media were the same as described previously (37, 38) unless indicated otherwise.  $\beta$ -Galactosidase assays were performed as described previously (38).

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Chelex-treated M17 was prepared by autoclaving 2% Chelex 100 resin (Bio-Rad) with M17, followed by 2 h of stirring. After removal of the resin, 50  $\mu$ M CaCl<sub>2</sub>, 50  $\mu$ M MgCl<sub>2</sub>, 5  $\mu$ M FeCl<sub>3</sub>, and 0.25% glucose were added, and the resulting medium, designated GM17chel, was used as growth medium as specified in Results. Metal ions were added as the salts ZnSO<sub>4</sub>, MnCl<sub>2</sub>, MgCl<sub>2</sub>, CaCl<sub>2</sub>, NiCl<sub>2</sub>, CuSO<sub>4</sub>, and FeSO<sub>4</sub>. The strains and plasmids used or constructed in this study are listed in Table 1. Primers are listed in Table 2. Since most work presented in this paper was carried out before publication of the D39 genome sequence (43), primer sequences are based on the R6 genome sequence (27).

**Construction of transcriptional *lacZ* fusions.** Ectopic *lacZ* fusions to the *pcpA* (spr1945), *psaR* (spr1480), *psaB* (spr1492), *nrdD* (spr0183), and spr0276 promoters were made in pPP2 with primer pairs pspr1945-1/pspr1945-2, marR-lacZ1/marR-lacZ2, PpsaB-1-lacZ/PpsaB-2-lacZ, Pspr183-1.2/Pspr183-2.2, and Pspr0267-1/Pspr0267-2, yielding plasmids pRW5, pRW4, pRW6, pRW3, and pRW2. A chromosomal *lacZ* fusion to the 3' end of *prtA* (spr0561) was made with primer pair spr0561-1/spr0561-2 in plasmid pORI13, leading to plasmid pRW1. In all cases *Escherichia coli* EC1000 was used as the cloning host. The *lacZ* fusion constructs were introduced into wild-type D39 and D39  $\Delta$ *psaR* (RW100) in the case of pPP2 (integration via double crossover in the *bgaA* gene) and into D39*nisRK* and its isogenic *psaR* mutant (RW101) in the case of pORI13 (integration by single crossover), giving strains RW102 to RW113. The *PcpA-lacZ* fusion was also introduced into the *psaCA* (RW121) and *czcD* (MP102) deletion strains, giving strains RW114 and RW122. All plasmid constructs were checked by sequencing, and new loci created with these plasmids were verified by PCR.

**Random mutagenesis screen.** Random mutagenesis using the *HimarI* MarC9 mariner transposon was performed essentially as described previously (42, 49). pR412-T7 (8), a derivative of pR412, was used as the source of the *spec* mariner transposon. Mutated R6 chromosomal DNA was transformed into strain R6, yielding a mutant library of approximately 20,000 CFU. Chromosomal DNA of this library was used to perform random mutagenesis in strain D39  $\Delta$ *bgaA::PcpA-lacZ*. Mutants were selected on GM17 with 1% sheep blood, 0.006% X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside), 1.5  $\mu$ g/ml tetracycline, and 130  $\mu$ g/ml spectinomycin. Transposon insertion sites in mutants showing derepression of expression of the *PcpA-lacZ* transcriptional fusion were identified by the direct sequencing method as described previously (34), using primer PBMrTn1 with PBMrIRPi (nested).

**Construction of deletion strains.** An in-frame marker-free deletion of *psaR* (spr1480) was constructed with plasmid pORI280 essentially as described previously (37), using primer pairs marR-del1/marR-del2 and marR-del3/marR-del4 and *E. coli* EC1000 as the cloning host. The mutant was checked by PCR and DNA sequencing.

A deletion strain of *psaCA* was made with primer pairs *psaCA*-KO-1/*psaCA*-KO-2 and *psaCA*-KO-1/*psaCA*-KO-4 by overlap extension PCR (73) and allelic replacement with an erythromycin resistance cassette, yielding strain RW121.

**Construction of *pcpA* promoter subclones in pPP2.** The following promoter subclones of the *pcpA* promoter were made in pPP2 (primer pairs are in parentheses): *PcpA*-3 (*PcpA*\_f\_2 and *PcpA*\_rev\_1), *PcpA*-3a (*PcpA*\_f\_3a and *PcpA*\_rev\_1), *PcpA*-4 (*PcpA*\_f\_3 and *PcpA*\_rev\_1), *PcpA*-5 (*PcpA*\_f\_4 and *PcpA*\_rev\_1), *PcpA*-rev3 (spr1945-1 and *PcpA*\_rev\_3), and *PcpA*-rev4 (spr1945-1 and *PcpA*\_rev\_4). All fragments were cloned as EcoRI/BamHI fragments in the same sites of pPP2, yielding plasmids pRW1 to pRW16. The constructs were sequenced and introduced into strains D39 and D39 $\Delta$ *psaR* (RW100), giving strains RW131 to RW136 and RW141 to RW146.

**Construction of point mutations in the *PsaR* binding site in *PcpA*.** A pNG8048E derivative containing a 64-bp fragment of the *pcpA* promoter comprising the *PsaR* binding site was constructed by annealing oligonucleotides P-*pcpA*-box1/P-*pcpA*-box2 and cloning them into NcoI/XbaI-digested pNG8048E, giving plasmid pRW21. This plasmid was used as a template for a PCR with Phusion DNA polymerase using the primer pairs *PcpA*\_mut1.1/*PcpA*\_mut1.2, *PcpA*\_mut2.1/*PcpA*\_mut2.2, and *PcpA*\_mut3.1/*PcpA*\_mut3.2, giving rise to plasmids pRW22 and pRW23, each with one point mutation in the predicted *PsaR* binding site, and pRW24, with two point mutations in the predicted *PsaR* binding site, respectively. The constructs were checked by DNA sequencing.

**Microarray analyses.** For microarray analysis, the transcriptome of wild-type D39 grown in four biological replicates in GM17 was compared to the transcriptome of the same strain grown in four biological replicates in GM17 plus 0.25 mM ZnSO<sub>4</sub>. Growth, RNA isolation, labeling, hybridization, and analysis of slides were done essentially as described previously (38). Since the analysis was performed by interslide comparisons (i.e., the wild-type transcriptome analyzed with four slides was compared to the wild-type transcriptome in GM17 plus Zn<sup>2+</sup> analyzed with four different slides), scaled signals from PostPrep of the slides hybridized to the GM17 samples were pairwise compared with the scaled signals

of the slides hybridized to the GM17-Zn<sup>2+</sup> samples. The resultant table with the scaled (and normalized) signals was used as input for the CyberT variant for statistical analysis of control versus experimental data, using a local running copy of the CyberT algorithm for paired data (<http://molgen.biol.rug.nl/cgi-bin/cybert/CyberT-8.0.form.pl?DATATYPE=CE>). Genes were considered differentially expressed when the Bayesian *P* value was <0.001 and the false discovery rate was <0.01, unless otherwise stated (80).

**Overexpression and purification of Strep-tagged *PsaR*.** For the overexpression of a C-terminally Strep-tagged variant of *PsaR*, *psaR* was amplified from D39 chromosomal DNA using primers Spr1480OX-1 and Spr1480-Ctermstrep\_OX. The resulting PCR product was digested with XbaI/XbaI and cloned into the NcoI/XbaI sites of pNG8048E, yielding plasmid pRW25. Overexpression in *Lactococcus lactis* NZ9000 was done essentially as described previously (41). Purification of *PsaR*-Strep from *L. lactis* was performed using the Streptactin column from IBA according to the supplier's instructions. Buffers without EDTA were used, and the purified protein was stored at a concentration of 0.15 mg/ml in the elution buffer (100 mM Tris-HCl [pH 8], 150 mM NaCl, 2.5 mM dethio-biotin, 1 mM  $\beta$ -mercaptoethanol) with 10% glycerol at -80°C.

**EMSAs.** Electrophoretic mobility shift assays (EMSAs) were performed with [ $\gamma$ -<sup>32</sup>P]ATP-labeled probes in buffer containing 20 mM Tris-HCl (pH 8.0), 5 mM MgCl<sub>2</sub>, 0.1 mM dithiothreitol, 8.7% (wt/vol) glycerol, 62.5 mM KCl, 25  $\mu$ g/ml bovine serum albumin, 25  $\mu$ g/ml poly(dI-dC), and 3000 cpm of [ $\gamma$ -<sup>32</sup>P]ATP-labeled PCR product. Various metal ions were added in concentrations as specified in Results. As probes, PCR products comprising the promoter regions of *psaB* (primers PpsaB-1-lacZ/PpsaB-2-lacZ), *pcpA* (*PcpA*\_f\_2/*PcpA*\_rev\_1), and *prtA* (*PprtA*-1/*PprtA*-2) were used. As a control, a PCR fragment of the *pcpA* promoter without the intact *PsaR* operator was used (*PcpA*\_f\_4/*PcpA*\_rev\_1). Reaction mixtures were incubated at 37°C for 10 min before loading on gels. Gels were run in 0.44 M Tris-borate buffer (pH 8.3) at 100 V for 90 min.

**Measurement of concentrations of Mn<sup>2+</sup> and Zn<sup>2+</sup> in growth media.** Using atomic absorption spectroscopy on a Vista AX-CCD simultaneous ICP-AES spectrometer, the concentrations of Mn<sup>2+</sup> and Zn<sup>2+</sup> in GM17 and GM17chel were determined at wavelengths (nm) of 257.610/259.372 (Mn) and 202.548/213.857 (Zn).

**Microarray accession number.** Microarray data have been deposited to the Gene Expression Omnibus (GEO) and can be accessed via <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE11438>. In addition, slide images and raw and processed data are available at [http://molgen.biol.rug.nl/publication/zinc\\_data/](http://molgen.biol.rug.nl/publication/zinc_data/).

## RESULTS

**Identification of Zn<sup>2+</sup>-regulated genes in *S. pneumoniae*.** To identify genes that are regulated by a high level of Zn<sup>2+</sup> in *S. pneumoniae*, we compared the transcriptome of wild-type D39 grown in GM17 with that of the same strain grown in GM17 with 0.25 mM ZnSO<sub>4</sub> (Table 3). With this concentration of Zn<sup>2+</sup>, growth is not affected (39). The most upregulated genes were *pcpA*, encoding a choline binding protein (69) involved in virulence (31), and Zn<sup>2+</sup> resistance gene *czcD* (39). Also upregulated were the serine protease gene *prtA* and the Mn<sup>2+</sup> ABC transporter genes *psaBC*, which are both involved in virulence (6, 7, 32, 50), and an operon (SP0202 to SP0207) encoding, among other proteins, the NrdDG ribonucleoside triphosphate reductase, which is involved in synthesis of deoxyribonucleoside triphosphates (33, 78). An operon consisting of genes involved in cellobiose metabolism (SP0303 to SP0310) (52) was strongly downregulated. Thus, expression of genes with a variety of functions is affected by growth of *S. pneumoniae* in medium with an elevated Zn<sup>2+</sup> concentration.

**Zn<sup>2+</sup>-dependent expression of *pcpA* is mediated by *PsaR*.** To investigate in more detail the transcriptional regulation of *pcpA*, the most Zn<sup>2+</sup>-induced gene found in the microarray experiment, a transcriptional *lacZ* fusion to the *pcpA* promoter was constructed by use of plasmid pPP2, which integrates into the *bgaA* locus (22). Using strain D39 containing this *PcpA-lacZ* transcriptional fusion, it was demonstrated that only ele-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description <sup>a</sup>	Reference or source
<i>S. pneumoniae</i>		
D39	Serotype 2 strain, <i>cps2</i>	4, 43; laboratory of P. Hermans
R6	D39 ( <i>cps2</i> 2538–9862) with increased transformation efficiency	27
D39 <i>nisRK</i>	D39 $\Delta bgaA::nisRK$ ; Trmp <sup>r</sup>	37
MP102	D39 $\Delta czcD$	This work
RW100	D39 $\Delta psaR$	This work
RW101	D39 <i>nisRK</i> $\Delta psaR$	This work
RW102	D39 $\Delta bgaA::PpcpA-lacZ$ ; Tet <sup>r</sup>	This work
RW103	D39 $\Delta bgaA::PnrdD-lacZ$ ; Tet <sup>r</sup>	This work
RW104	D39 <i>nisRK</i> <i>prtA-lacZ</i> ; Erm <sup>r</sup>	This work
RW105	D39 $\Delta bgaA::Pspr0276-lacZ$ ; Tet <sup>r</sup>	This work
RW106	D39 $\Delta bgaA::PpsaR-lacZ$ ; Tet <sup>r</sup>	This work
RW107	RW100 $\Delta bgaA::PpcpA-lacZ$ ; Tet <sup>r</sup>	This work
RW108	RW100 $\Delta bgaA::PnrdD-lacZ$ ; Tet <sup>r</sup>	This work
RW109	RW101 <i>prtA-lacZ</i> ; Erm <sup>r</sup>	This work
RW110	RW100 $\Delta bgaA::Pspr0276-lacZ$ ; Tet <sup>r</sup>	This work
RW111	RW100 $\Delta bgaA::PpsaR-lacZ$ ; Tet <sup>r</sup>	This work
RW112	D39 $\Delta bgaA::PpsaB-lacZ$ ; Tet <sup>r</sup>	This work
RW113	RW100 $\Delta bgaA::PpsaB-lacZ$ ; Tet <sup>r</sup>	This work
RW114	RW121 $\Delta bgaA::PpcpA-lacZ$ ; Tet <sup>r</sup>	This work
RW120	RW103 with mariner insertion in <i>psaR</i> ; Spec <sup>r</sup>	This work
RW121	D39 $\Delta psaCA::ermR$ ; Erm <sup>r</sup>	This work
RW122	MP102 $\Delta bgaA::PpcpA-lacZ$ ; Tet <sup>r</sup>	This work
RW131	D39 $\Delta bgaA::PpcpA-3-lacZ$ ; Tet <sup>r</sup>	This work
RW132	D39 $\Delta bgaA::PpcpA-3a-lacZ$ ; Tet <sup>r</sup>	This work
RW133	D39 $\Delta bgaA::PpcpA-4-lacZ$ ; Tet <sup>r</sup>	This work
RW134	D39 $\Delta bgaA::PpcpA-5-lacZ$ ; Tet <sup>r</sup>	This work
RW135	D39 $\Delta bgaA::PpcpA-rev3-lacZ$ ; Tet <sup>r</sup>	This work
RW136	D39 $\Delta bgaA::PpcpA-rev4-lacZ$ ; Tet <sup>r</sup>	This work
RW141	RW100 $\Delta bgaA::PpcpA-3-lacZ$ ; Tet <sup>r</sup>	This work
RW142	RW100 $\Delta bgaA::PpcpA-3a-lacZ$ ; Tet <sup>r</sup>	This work
RW143	RW100 $\Delta bgaA::PpcpA-4-lacZ$ ; Tet <sup>r</sup>	This work
RW144	RW100 $\Delta bgaA::PpcpA-5-lacZ$ ; Tet <sup>r</sup>	This work
RW145	RW100 $\Delta bgaA::PpcpA-rev3-lacZ$ ; Tet <sup>r</sup>	This work
RW146	RW100 $\Delta bgaA::PpcpA-rev4-lacZ$ ; Tet <sup>r</sup>	This work
<i>E. coli</i> EC1000	Km <sup>r</sup> ; MC1000 derivative carrying a single copy of the pWV01 <i>repA</i> gene in <i>glgB</i>	45
<i>L. lactis</i> NZ9000	MG1363 $\Delta pepN::nisRK$	41
Plasmids		
pR412T7	Spec <sup>r</sup> ; derivative of pR412 (49)	8
pORI13	Erm <sup>r</sup> ; <i>ori</i> <sup>+</sup> <i>repA</i> <sup>+</sup> ; promoterless <i>lacZ</i> , for single-copy chromosomal <i>lacZ</i> fusions.	70
pORI280	Erm <sup>r</sup> ; <i>ori</i> <sup>+</sup> <i>repA</i> <sup>+</sup> ; deletion derivative of pWV01; constitutive <i>lacZ</i> expression from P32 promoter	45
pPP2	Amp <sup>r</sup> Tet <sup>r</sup> ; promoterless <i>lacZ</i> ; for replacement of <i>bgaA</i> ( <i>spr0565</i> ) with promoter- <i>lacZ</i> fusions; derivative of pPP1	22
pNZ8048	Cm <sup>r</sup> ; nisin-inducible <i>PnisA</i>	16
PNG8048E	Cm <sup>r</sup> Erm <sup>r</sup> ; nisin-inducible <i>PnisA</i> , pNZ8048 derivative containing Erm <sup>r</sup> gene to facilitate cloning	Laboratory collection
pRW1	pORI13:: <i>prtA-lacZ</i>	This work
pRW2	pPP2 <i>Pspr0276</i>	This work
pRW3	pPP2 <i>PnrdD</i>	This work
pRW4	pPP2 <i>PpsaR</i>	This work
pRW5	pPP2 <i>PpcpA</i>	This work
pRW6	pPP2 <i>PpsaB</i>	This work
pRW11	pPP2 <i>PpcpA-3</i>	This work
pRW12	pPP2 <i>PpcpA-3a</i>	This work
pRW13	pPP2 <i>PpcpA-4</i>	This work
pRW14	pPP2 <i>PpcpA-5</i>	This work
pRW15	pPP2 <i>PpcpA-rev3</i>	This work
pRW16	pPP2 <i>PpcpA-rev4</i>	This work
pRW20	pORI280 $\Delta psaR$	This work
pRW21	pNG8048E containing a 64-bp fragment comprising the <i>PsaR</i> binding site	This work
pRW22	pRW12 containing a point mutation (T→G, bp –186 <sup>b</sup> ) in the <i>PsaR</i> binding box	This work
pRW23	pRW12 containing a point mutation (A→G, bp –184 <sup>b</sup> ) in the <i>PsaR</i> binding box	This work
pRW24	pRW12 containing two point mutations (A→G, bp –184 <sup>b</sup> ; A→C, bp –185 <sup>b</sup> ) in the <i>PsaR</i> binding box	This work
pRW25	pNG8048E carrying <i>psaR-strep</i> downstream of <i>PnisA</i>	This work

<sup>a</sup> Erm<sup>r</sup>, erythromycin resistance; Tet<sup>r</sup>, tetracycline resistance; Cm<sup>r</sup>, chloramphenicol resistance; Spec<sup>r</sup>, spectinomycin resistance.<sup>b</sup> Where the first base of the *pepA* start codon (ATG) is +1.



TABLE 2. Oligonucleotide primers used in this study

Primer	Nucleotide sequence (5' to 3')	Restriction site
PBMrIRPi	AGACCGGGGACTTATCAGCC	
PBMrTn1	CTAGCGACGCCATCTATGTG	
TMr_1	TGCATTTAATACTAGCGACGCCATCTATGTGTC	
TMr_4	GGATCCATTCGCGTCAATTCGAGGGG	
PpsaB-1-lacZ	CGGAATTCTTCCAAGTTTTTACACTTG	EcoRI
PpsaB-2-lacZ	CGGGATCCATTGTTGGTCCATGGAGCAC	BamHI
pspr1945-1	CGGAATTCCTTCAAATTTTAAAGTCC	EcoRI
pspr1945-2	CGGGATCCGTTAATGATAATATTGTAG	BamHI
PpcpA_f_2	CGGAATTCTAATTTCTTTTAAACCCAC	EcoRI
PpcpA_f_3	CGGAATTCGTGGGTAAATTTTCTTGAC	EcoRI
PpcpA_f_3a	CGGAATTCATAAAAAAGAAATTAAGTGG	EcoRI
PpcpA_f_4	CGGAATTCATGTACTACTCTATTCTAC	EcoRI
PpcpA_rev_1	CGGGATCCAGGATTGGTTCTATAGGGAC	BamHI
PpcpA_rev_3	CGGGATCCGTCAAGGAAAATTAACCCAC	BamHI
PpcpA_rev_4	CGGGATCCCTAGTAGAATAGAGTAGTAC	BamHI
marR-lacZ1	CGGAATTCGCTATTTTCGTCAATCC	EcoRI
marR-lacZ2	CGGGATCCCATTTTATAGATCTTCTTTG	BamHI
marR-del1	TGCTCTAGACAATTGCCACCAGTCCCG	XbaI
marR-del2	TTCTTTGTTTGGGGTCATTC	
marR-del3	GACCCCAACAAAGAAGTCGAGAAAATCAACTAAT	
marR-del4	GAAGATCTCTTTTGTCTAGCTGAACGA	BglII
PprtA-1	CGGAATTCAGCGCTGATATTTTCATC	
PprtA-2	CGGGATCCGTTAGTGACAATACTGTG	
spr0561-1	CGGAATTCGATACGGGAGAGGTAAGTG	EcoRI
spr0561-2	CGGGATCCGAAATTGTCTCATCACCTC	BamHI
PSpr183-1.2	CGGAATTCACCTAAGGTGATTGTGG	EcoRI
PSpr183-2.2	CGGGATCCGAATTTCTGTAATAATTCGC	BamHI
Pspr0276-1	CGGAATTCGAATTAGAATTATTGTGAG	BamHI
Pspr0276-2	CGGGATCCGAGCAGCAACAGCACCACC	XbaI
Ery-for	TAACGATTATGCCGATAACT	
Ery-rev	GCATGCATCGATTAGATCTC	
psaCA-KO-1	AAATCCTTACACCGAATTGC	
psaCA-KO-2	GAGATCTAATCGATGCATGCTTCTGCAATCATAGGTCACCTCC	
psaCA-KO-3	AGTTATCGGCATAATCGTTAGACAAGATTGCTGAAGGATTGG	
psaCA-KO-4	TGCGCGTGCTAATAGGTGCC	
P-pcpA-box1	CATGTCTTTTTTAACACGGGTTAAAAAAGAAATTAAAGTGGGTTAATTTTCCTTGACTT AAAAATTTAA	
P-pcpA-box2	CTAGTTAAATTTTAAGTCAAGGAAAATTAACCCACTTTAATTTCTTTTTTAACCCGTGT TAAAAAAGA	
PcpA_mut1.1	GATTTCCTTTTTTAACCCGTG	
PcpA_mut1.2	AAAGTGGGTAAATTTTCCTTG	
PpcpA_mut2.1	GTAATTTCTTTTTTAACCCGTG	
PpcpA_mut2.2	AGTGGGTAAATTTTCCTTGAC	
PpcpA_mut3.1	GCAATTTCTTTTTTAACCCGTG	
PpcpA_mut3.2	AGTGGGTAAATTTTCCTTGAC	
Spr1480OX-2	TGCTCTAGATTAGTTGATTTTCTCGACATAG	XbaI
spr1480-OX-new	CGAGCCATCATGAAACATCATCATCATCATACCCCAAACAAAGAAGACTATC	RcaI
Spr1480-Ctermstrep_OX	TGCTCTAGATTATTTTCAAATTGTGGATGGCTCCAAGCGCTGTTGATTTTCTCGACAT AGAGTTG	XbaI
Spr1480OX-1	CGAGCCATCATGACCCCAAACAAAGAAGAC	RcaI

vated  $Zn^{2+}$  concentrations lead to high expression from the *pcpA* promoter, although also some weak induction could be seen for  $Co^{2+}$  and  $Fe^{2+}$  (Table 4).

To identify the factor repressing transcription of *pcpA* under normal growth conditions, strain D39 *PpcpA-lacZ* (RW102) was randomly mutagenized with the mariner transposon (42, 49) and blue colonies were screened for on GM17 agar plates with X-Gal but without  $Zn^{2+}$  supplementation. Among 7,200 CFU, one blue clone was found, containing a transposon insertion in the gene encoding the MarR family transcriptional regulator PsaR (spr1480, SPD\_1450, SP1638). The insertion in *psaR* gave rise to high expression of the *PpcpA-lacZ* fusion, which was independent of  $Zn^{2+}$  (data not shown). We con-

structed a markerless deletion mutant of *psaR* and found that in this mutant expression of *PpcpA-lacZ* is highly derepressed independent of  $Zn^{2+}$  (Table 5), suggesting that PsaR is responsible for  $Zn^{2+}$ -dependent derepression of *pcpA* expression.

**PsaR regulates *prtA* and *psaBCA* in a  $Zn^{2+}$ -dependent way.** To test whether PsaR is also responsible for the  $Zn^{2+}$ -dependent expression of the other genes identified in the microarray analysis, transcriptional *lacZ* fusions to *prtA*, *PpsaB*, and *PnrdD* (Pspr0183) were constructed and introduced in both the wild type and the *psaR* mutant. As expected, in the wild type expression of *prtA* and *PpsaB* increased upon addition of  $Zn^{2+}$  to the GM17 growth medium (Tables 6 and 7). In the *psaR*

TABLE 3. Summary of transcriptome comparison of *S. pneumoniae* strain D39 grown in GM17 and in GM17 with addition of 0.25 mM Zn<sup>2+</sup>

TIGR4 locus tag	Function (TIGR annotation)	Ratio <sup>a</sup>
SP0202	Anaerobic ribonucleoside triphosphate reductase NrdD	2.5
SP0204	Predicted acetyltransferase, GNAT family	2.4
SP0205	Anaerobic ribonucleoside triphosphate reductase activating protein NrdG	1.8 <sup>b</sup>
SP0206	Hypothetical protein; predicted uridine kinase	1.8 <sup>b</sup>
SP0207	Hypothetical protein; predicted uridine kinase	2.2
SP0303	6-Phospho-β-glucosidase BglA	−7.6
SP0305	Phosphotransferase system cellobiose-specific component IIB	−4.1
SP0306	Putative transcriptional regulator; possible antiterminator BglG	−14.8
SP0307	Phosphotransferase system, IIA component	−6.3 <sup>b</sup>
SP0308	Phosphotransferase system cellobiose-specific component IIA	−3.0
SP0309	Hypothetical protein	−7.6
SP0310	Phosphotransferase system cellobiose-specific component IIC	−6.0
SP0338	Putative ATP-dependent <i>clp</i> protease, ATP binding subunit ClpL	−2.0
SP0515	Heat-inducible transcription repressor HrcA	−2.0
SP0516	Heat shock protein GrpE	−1.6
SP0517	Chaperone protein DnaK (heat shock protein 70)	−1.4
SP0518	Hypothetical protein	−2.1
SP0519	Chaperone protein DnaJ	−1.4
SP0640	Hypothetical protein	1.5 <sup>b</sup>
SP0641	Cell wall-associated serine proteinase precursor PrtA	2.7
SP0645	Putative phosphotransferase system IIA component	2.8
SP0646	Phosphotransferase system, IIB component, putative	2.4
SP0879	Hypothetical protein	−2.1
SP1648	Manganese (and/or zinc) ABC transporter, ATP binding protein PsaB	2.3
SP1649 <sup>c</sup>	Manganese (and/or zinc) ABC transporter, permease protein PsaC	2.6
SP1762	Hypothetical protein	2.7
SP1855	Alcohol dehydrogenase, zinc-containing AdhB	1.7
SP1856	Transcriptional regulator, MerR family	2.0 <sup>b</sup>
SP1857	Cation efflux system protein CzcD	7.2
SP1935	Hypothetical protein	2.8
SP2136	Choline binding protein; surface protein PcpA	8.5

<sup>a</sup> Ratios of >2.0 or <−2.0 (wild-type D39 compared to wild-type D39 plus 0.25 mM Zn<sup>2+</sup>) are shown; in some cases neighboring genes with lower fold changes are also indicated.  
<sup>b</sup> Ratio with a false discovery rate of >0.01 but <0.1.  
<sup>c</sup> For SP1650 (*psaA*), the number of observations (replicates) was too low and hence no significance was obtained.

mutant expression was derepressed in GM17, showing that PsaR mediates the Zn<sup>2+</sup>-dependent expression of *prtA* and *psaBCA* as well. Transcription from *PnrdD* was two- to three-fold higher in cells grown in GM17 with Zn<sup>2+</sup> compared to GM17, but this was not affected by the *psaR* mutation (data not shown). We also tested the effect of metal ions on the expres-

sion of *psaR* itself. A *PpsaR-lacZ* transcriptional fusion was highly expressed in GM17 but was not influenced by Zn<sup>2+</sup> and Mn<sup>2+</sup> or by the *psaR* mutation (data not shown).

The expression of the cellobiose utilization operon SP303 to SP310 (*spr0276* to *spr0282*, R6 annotation) was further analyzed using a *Pspr0276-lacZ* transcriptional fusion. Surprisingly, in GM17 the expression was hardly measurable, and no effect of Zn<sup>2+</sup> was observed (data not shown). However, in the absence of glucose as well as in the presence of cellobiose, *Pspr0276* was highly expressed (data not shown). It is not immediately clear what the reason is for the downregulation of this operon in the transcriptome analysis.

**Regulation of *PpsaB*, *PpcpA*, and *prtA* depends on the balance between Mn<sup>2+</sup> and Zn<sup>2+</sup>.** In an earlier study, expression of *pcpA* and *psaBCA* was demonstrated to be repressed by PsaR in the presence of Mn<sup>2+</sup> (31). To specify how Mn<sup>2+</sup> and Zn<sup>2+</sup> influence PsaR activity, we analyzed the expression of transcriptional *lacZ* fusions to *PpcpA*, *PpsaB*, and *prtA* in the presence of various concentrations of both Mn<sup>2+</sup> and Zn<sup>2+</sup> in GM17 medium and in GM17 medium treated with the metal ion chelator Chelex 100 resin (GM17chel). GM17 contains 2.0 μM Mn<sup>2+</sup> and 8.1 μM Zn<sup>2+</sup> and GM17chel contains 0.02 μM Mn<sup>2+</sup> and 0.00 μM Zn<sup>2+</sup>, showing that the Chelex treatment effectively removed these cations. In GM17, upregulation of the expression of all three *lacZ* fusions in the presence of a high concentration of Zn<sup>2+</sup> was nullified by the addition of

TABLE 4. Expression of <i>PpcpA</i> specifically in the presence of various metal cations <sup>a</sup>	
Addition to GM17 (mM)	Mean (SD) β-galactosidase activity (Miller units)
None	4 (1)
Zn <sup>2+</sup> (0.2)	54 (12)
Zn <sup>2+</sup> (0.4)	388 (45)
Cu <sup>2+</sup> (0.05)	3 (1)
Cu <sup>2+</sup> (0.1)	3 (1)
Co <sup>2+</sup> (0.05)	8 (3)
Co <sup>2+</sup> (0.1)	16 (3)
Ni <sup>2+</sup> (0.1)	4 (1)
Ni <sup>2+</sup> (0.4)	5 (2)
Fe <sup>2+</sup> (0.1)	9 (2)
Fe <sup>2+</sup> (0.4)	21 (6)
Mg <sup>2+</sup> (1.0)	4 (1)
Mg <sup>2+</sup> (10)	2 (1)

<sup>a</sup> β-Galactosidase of a *PpcpA-lacZ* transcriptional fusion was measured in the wild-type strain D39 (strain RW102) grown in GM17 with the indicated metal ions. For Zn<sup>2+</sup>, Ni<sup>2+</sup>, Co<sup>2+</sup>, and Cu<sup>2+</sup> the concentrations used have similar effects on growth (see also reference 39). Values are from three experiments.

TABLE 5.  $\beta$ -Galactosidase activities of the wild-type D39 and the  $\Delta$ *psaR*,  $\Delta$ *psaCA*, and  $\Delta$ *czcD* strains, all containing the *PpcpA-lacZ* transcriptional fusion, grown in GM17 and GM17chel supplemented with metal ions<sup>a</sup>

Medium and addition(s) (mM)	Mean (SD) $\beta$ -galactosidase activity (Miller units) <sup>b</sup> in:			
	Wild type	$\Delta$ <i>psaR</i> mutant	$\Delta$ <i>psaCA</i> mutant	$\Delta$ <i>czcD</i> mutant
GM17				
None	6 (3)	1,204 (67)	594 (55)	18 (5)
Zn <sup>2+</sup> (0.1)	17 (4)	ND	ND	69 (12)
Zn <sup>2+</sup> (0.2)	66 (21)	1,102 (277)	ND	ND
Zn <sup>2+</sup> (0.4)	417 (113)	1,189 (115)	446 (37)	ND
Zn <sup>2+</sup> (0.4) + Mn <sup>2+</sup> (0.01)	33 (12)	1,119 (113)	237 (22)	ND
Zn <sup>2+</sup> (0.4) + Mn <sup>2+</sup> (0.05)	11 (4)	1,219 (203)	7 (4)	ND
Zn <sup>2+</sup> (0.4) + Fe <sup>2+</sup> (0.05)	269 (22)	ND	ND	ND
GM17chel				
None	315 (47)	1,217 (222)	ND	ND
Zn <sup>2+</sup> (0.2)	424 (35)	1,260 (102)	ND	ND
Mn <sup>2+</sup> (0.05)	6 (2)	1,315 (78)	ND	ND

<sup>a</sup> The wild-type D39 and the  $\Delta$ *psaR*,  $\Delta$ *psaCA*, and  $\Delta$ *czcD* strains are strains RW102, RW107, RW114, and RW122, respectively.  
<sup>b</sup> Values are from three experiments. ND, not determined.

Mn<sup>2+</sup> when added in a concentration of 0.01 to 0.05 mM, which is about 10 to 40 times lower than the concentration of Zn<sup>2+</sup> (Tables 5, 6, and 7). This repressive effect was specific for Mn<sup>2+</sup>, since 0.05 mM Ni<sup>2+</sup>, Cu<sup>2+</sup>, and Co<sup>2+</sup> had no effect on the Zn<sup>2+</sup>-dependent expression of the *PpcpA-lacZ* transcriptional fusion (data not shown); for Fe<sup>2+</sup>, there was only a weak repressive effect (Table 5). In GM17chel, expression of all three *lacZ* fusions was derepressed compared to that in GM17 (Tables 5, 6, and 7), which was expected because of the much lower concentration of Mn<sup>2+</sup> after Chelex treatment. Addition of Zn<sup>2+</sup> increased the derepression even more, while Mn<sup>2+</sup> led to repression of expression again, which is in agreement with the observations made in GM17 (Tables 5, 6, and 7). To investigate whether the opposite effects of Zn<sup>2+</sup> and Mn<sup>2+</sup> are the result of the competition for uptake of these cations, expression of the *PpcpA-lacZ* fusion in *czcD* and *psaCA* deletion mutants was measured. In a *czcD* deletion

mutant, which is, as a consequence of impaired Zn<sup>2+</sup> efflux, likely to accumulate higher intracellular levels of this metal ion (39), expression of *PpcpA-lacZ* in both GM17 and GM17 with 0.1 mM Zn<sup>2+</sup> (the highest possible concentration for the *czcD* deletion mutant) was higher than that in the wild type (Table 5). In a *psaCA* deletion mutant, which is impaired in uptake of Mn<sup>2+</sup> into the cell (50) the expression of *PpcpA-lacZ* was also highly derepressed. However, addition of Mn<sup>2+</sup>, albeit at a higher concentration than with the wild type, still led to repression (Table 5). These results suggest that the observed regulatory effects on expression of *pcpA*, *prtA*, and *psaBCA* that are induced by Mn<sup>2+</sup> and Zn<sup>2+</sup> converge at the level of transcriptional regulation by PsaR. **Identification of a PsaR operator in the promoters of *pcpA*, *prtA*, and *psaB*.** Using Gibbs Motif Sampler (77), a palindromic sequence (Fig. 1A and B), located just upstream of (*PprtA* and *PpcpA*) or overlapping with (*PpsaB*) the predicted core promoter regions, that might serve as the PsaR operator was uncovered.

To dissect the promoter of *pcpA* experimentally, we per-

TABLE 6.  $\beta$ -Galactosidase activities of the wild-type D39 and the  $\Delta$ *psaR* strain, containing the *prtA-lacZ* transcriptional fusion, grown in GM17 and GM17chel supplemented with metal ions<sup>a</sup>

Medium and addition(s) (mM)	Mean (SD) $\beta$ -galactosidase activity (Miller units) <sup>b</sup> in:	
	Wild type	$\Delta$ <i>psaR</i> mutant
GM17		
None	0.2 (0.06)	2.2 (0.3)
Zn <sup>2+</sup> (0.2)	0.6 (0.17)	2.3 (0.5)
Zn <sup>2+</sup> (0.4)	1.2 (0.05)	2.1 (0.4)
Zn <sup>2+</sup> (0.4) + Mn <sup>2+</sup> (0.01)	0.5 (0.09)	2.1 (0.2)
Zn <sup>2+</sup> (0.4) + Mn <sup>2+</sup> (0.05)	0.2 (0.08)	2.1 (0.2)
GM17chel		
None	0.5 (0.03)	2.2 (0.1)
Zn <sup>2+</sup> (0.2)	0.9 (0.05)	2.0 (0.4)
Mn <sup>2+</sup> (0.05)	0.2 (0.04)	2.1 (0.4)

<sup>a</sup> The wild-type D39 and the  $\Delta$ *psaR* strain are strains RW104 and RW109, respectively. *lacZ* was fused to the 3' end of *prtA* on the native chromosomal location, using plasmid pORI13. This might explain the much lower  $\beta$ -galactosidase activity compared to the values for the *lacZ* fusions with *PpcpA* and *PpsaB* (Tables 5 and 7).  
<sup>b</sup> Values are from three experiments.

TABLE 7.  $\beta$ -Galactosidase activities of the wild-type D39 and the  $\Delta$ *psaR* strain, containing the *PpsaB-lacZ* transcriptional fusion, grown in GM17 and GM17chel supplemented with metal ions<sup>a</sup>

Medium and addition(s) (mM)	Mean (SD) $\beta$ -galactosidase activity (Miller units) <sup>b</sup> in:	
	Wild type	$\Delta$ <i>psaR</i> mutant
GM17		
None	134 (34)	1,167 (121)
Zn <sup>2+</sup> (0.2)	448 (110)	970 (78)
Zn <sup>2+</sup> (0.4)	760 (124)	1,089 (98)
Zn <sup>2+</sup> (0.4) + Mn <sup>2+</sup> (0.01)	651 (18)	1,205 (144)
Zn <sup>2+</sup> (0.4) + Mn <sup>2+</sup> (0.05)	142 (11)	1,072 (262)
GM17chel		
None	583 (41)	1,107 (220)
Zn <sup>2+</sup> (0.2)	901 (33)	1,180 (102)
Mn <sup>2+</sup> (0.05)	111 (27)	1,023 (45)

<sup>a</sup> The wild-type D39 and the  $\Delta$ *psaR* strain are strains RW112 and RW113, respectively.  
<sup>b</sup> Values are from three experiments.

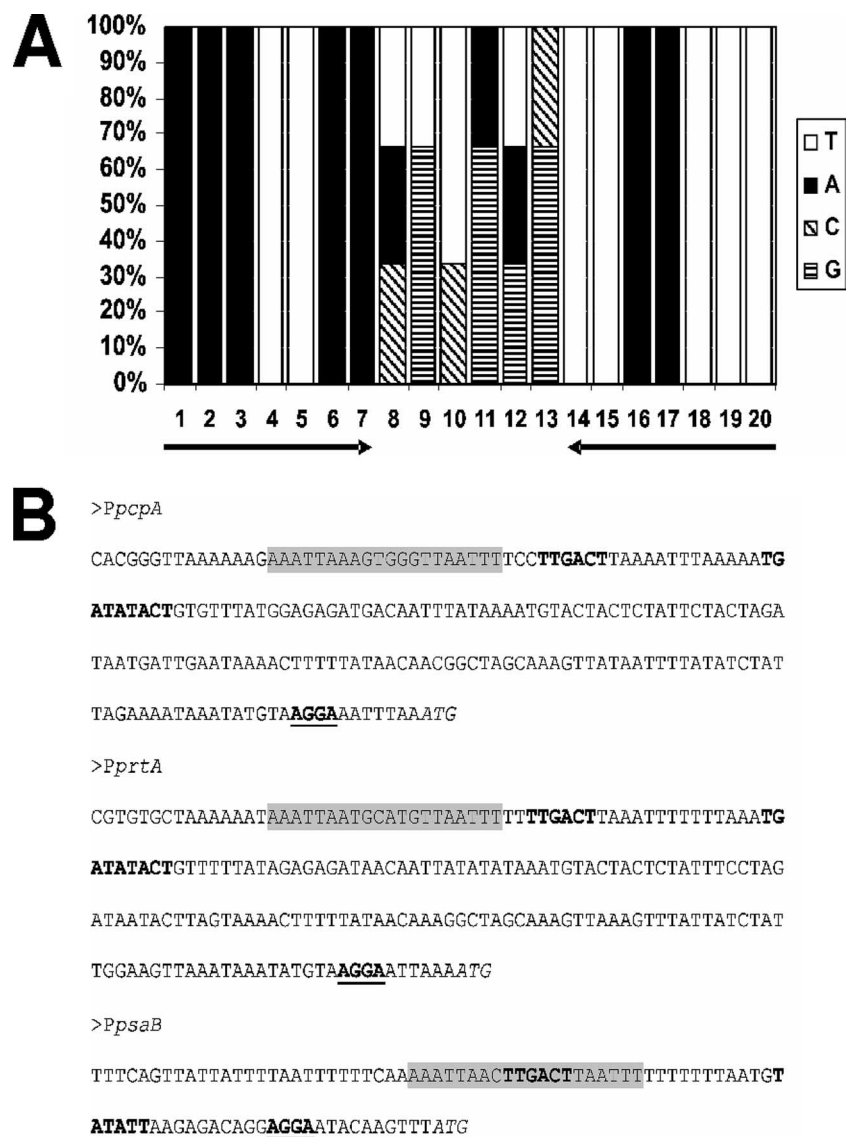


FIG. 1. Identification of a putative Psar operator. (A) Weight matrix of the identified Psar operator as present in the promoter regions of *pcpA*, *prtA*, and *psaB*. (B) Positions of the Psar operator (shaded) in the promoter regions of *pcpA*, *prtA*, and *psaB*. Putative core promoter sequences are in bold. The ribosome binding sites are in bold and underlined. Start codons are in italic.

formed a promoter subcloning experiment where the *pcpA* promoter was truncated from the 5' end and fused to *lacZ* in the reporter plasmid pPP2 (Fig. 2). Expression of a promoter fragment truncated upstream of the predicted operator (*PpcpA*-3) was Zn<sup>2+</sup> dependent, but as expected, deletion of half of the identified operator (*PpcpA*-4) led to fully derepressed, Zn<sup>2+</sup>-independent expression (Fig. 2). In the presence of 0.05 mM Mn<sup>2+</sup>, the β-galactosidase activity of *PpcpA*-4

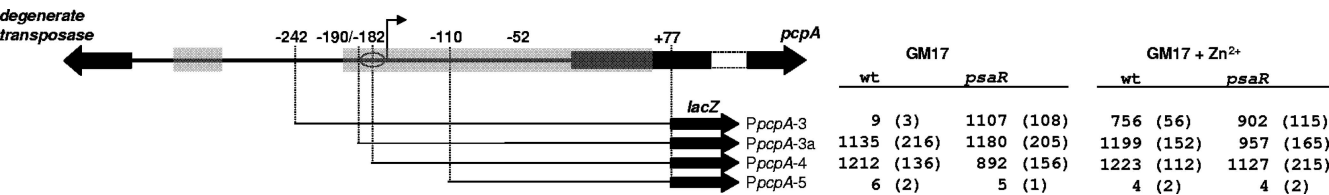


FIG. 2. Subcloning of *PpcpA*. A schematic overview of the *PpcpA* truncations is shown. Numbers indicate the positions of the truncations, which were fused to *lacZ*, relative to the putative *pcpA* start site (+1). The flag indicates the position of the core promoter, and the oval indicates the putative Psar operator. Gray-shaded areas indicate regions of similarity with *PprtA* (89% identity for the short stretch and 82% identity for the long stretch). The table on the right gives β-galactosidase activities (Miller units) of the promoter truncations in wild-type D39 (wt, strains RW131 to RW134) and the *ΔpsaR* mutant (*psaR*, strains RW141 to RW144) grown in GM17 and in GM17 plus 0.5 mM Zn<sup>2+</sup>. Standard deviations of three measurements are given in parentheses.



TABLE 8. Mutational analysis of the PsaR operator

PsaR box	Sequence (5'→3') <sup>a</sup>	Mean (SD) β-galactosidase activity (Miller units) for the following strain and medium <sup>b</sup> :			
		Wild type		Δ <i>psaR</i> mutant	
		GM17	GM17 + Zn <sup>2+</sup> (0.4 mM)	GM17	GM17 + Zn <sup>2+</sup> (0.4 mM)
Wild type	AAATTAAAGTGGGTTAATTT	733 (123)	853 (122)	1,288 (170)	1,331 (167)
mut 1	AAAT <b>G</b> AAAGTGGGTTAATTT	74 (15)	376 (78)	1,160 (135)	1,271 (122)
mut 2	AAATT <b>A</b> GAGTGGGTTAATTT	121 (25)	416 (75)	1,300 (101)	1,278 (69)
mut 3	AAATT <b>C</b> GAGTGGGTTAATTT	58 (12)	464 (16)	1,389 (111)	1,378 (178)

<sup>a</sup> Sequences of wild-type and mutant PsaR boxes of the *pcpA* promoter; point mutations are in bold.  
<sup>b</sup> β-Galactosidase activities of wild-type D39 and the Δ*psaR* mutant harboring the *PpcpA-lacZ* transcriptional fusion (strains RW102 and RW107) containing plasmid pRW21 (wild-type PsaR box), pRW22 (PsaR box mut1), pRW23 (PsaR box mut2), or pRW24 (PsaR box mut3).

in wild-type D39 was 1,197 ± 78 Miller units, which is similar to the values for *PpcpA*-4 in GM17 and GM17 with Zn<sup>2+</sup> (Fig. 2), indicating that the effects of Zn<sup>2+</sup> and Mn<sup>2+</sup> are mediated by the same PsaR operator site. To determine if the operator sequence was fully identified, a truncation of the promoter region 8 bp upstream of the operator sequence (*PpcpA*-3a) was constructed, which gave rise to full derepression, suggesting that additional bases 5' to the operator are also important for PsaR-mediated repression of the *pcpA* promoter. For subclone *PpcpA*-5, expression was close to zero under all conditions. Deletion of the same region as in *PpcpA*-5 but now from the 3' side (*PpcpA*-rev3 versus *PpcpA*-rev4; strains RW135, RW136, and RW145, RW146) confirmed that promoter activity locates exclusively to this area (data not shown). This demonstrates that the core promoter sequence is located in the region between the 5' base pair positions of subclones *PpcpA*-4 and *PpcpA*-5 (Fig. 2 and 1B).

To further show that the predicted PsaR operator is functional, a 64-bp DNA fragment of the *pcpA* promoter comprising the PsaR operator was put into plasmid pNG8048E, which replicates in *S. pneumoniae* (37). Subsequently, several point mutations in the first half of the motif were introduced (Table 8). By putting the wild-type construct into D39 containing the *PpcpA-lacZ* transcriptional fusion, transcription from *PpcpA* was strongly derepressed, showing that this 64-bp stretch of DNA titrates away the repressive effect of PsaR on the expression of *pcpA* (Table 8). However, with the constructs containing the mutated PsaR boxes, this derepressive effect was not present (Table 8). This shows clearly that the bases in the predicted PsaR binding box are required for PsaR-dependent repression of *pcpA*. The entire *S. pneumoniae* R6, D39, and TIGR4 sequences were searched with a weight matrix of the PsaR operator sequence (Fig. 1A) using Genome2D (5), but the motif was not found in additional promoter regions (data not shown). In conclusion, a PsaR regulatory element in the promoters of *pcpA*, *psaB*, and *prtA* was identified.

**Binding of PsaR-Strep to the *pcpA*, *psaB*, and *prtA* promoters in the presence of Mn<sup>2+</sup> is counteracted by Zn<sup>2+</sup>.** To find out whether the observed Zn<sup>2+</sup>- and Mn<sup>2+</sup>-dependent effects on expression of the PsaR targets are caused by direct modulation of the DNA binding activity of the PsaR protein, EMSAs were performed with purified Strep-tagged PsaR (PsaR-Strep). PsaR-Strep alone did not shift the promoter regions of *pcpA*, *psaB*, and *prtA* (Fig. 3A to C, lanes 2). However, in the presence of Mn<sup>2+</sup>, PsaR-Strep did bind to the promoter re-

gions of *pcpA*, *psaB*, and *prtA* (Fig. 3A to C, lanes 3). PsaR-Strep did not bind under any condition to a truncated *pcpA* promoter lacking the PsaR binding box (Fig. 3D). Besides Mn<sup>2+</sup>, 0.05 mM Co<sup>2+</sup> was also able to stimulate the binding of PsaR-Strep to the promoter fragments, whereas 0.05 mM Fe<sup>2+</sup>, Cu<sup>2+</sup>, Ni<sup>2+</sup>, and Zn<sup>2+</sup> did not (data not shown). These data show that the PsaR-Strep–DNA interaction was specific and indicates that PsaR directly functions as a Mn<sup>2+</sup>-dependent repressor of its target genes. Based on the *lacZ* expression studies, we hypothesized that Zn<sup>2+</sup> should somehow impair PsaR binding to fulfill its function as an Mn<sup>2+</sup>-dependent repressor. Therefore, experiments addressing the influence of Zn<sup>2+</sup> on the in vitro PsaR-Strep–DNA interaction in the presence of Mn<sup>2+</sup> were performed (Fig. 3A, B, and C, lanes 4 to 8). These demonstrated that the stimulatory effect of Mn<sup>2+</sup> on the binding of PsaR-Strep to all three promoter fragments was counteracted by the addition of Zn<sup>2+</sup>. There was also a weaker counteracting effect of Cu<sup>2+</sup> for the *pcpA* and *prtA* promoters (Fig. 3A and C, lanes 8). Thus, Mn<sup>2+</sup> stimulates PsaR binding to the operators in the *pcpA*, *psaB*, and *prtA* promoters, whereas in the presence of Zn<sup>2+</sup>, PsaR binding is abolished, indicating that Mn<sup>2+</sup> and Zn<sup>2+</sup> exert their regulatory effects on *pcpA*, *prtA*, and *psaBCA* expression directly through PsaR.

DISCUSSION

In this study, we analyzed the transcriptome change of the human pathogen *S. pneumoniae* in response to a high level of Zn<sup>2+</sup>. Expression of several genes and operons with diverse functions was affected by Zn<sup>2+</sup>, including *pcpA*, *prtA*, and *psaBCA*. The observed Zn<sup>2+</sup>-dependent expression of these virulence genes was shown to be directly mediated by the Mn<sup>2+</sup>-responsive repressor PsaR (31). We further demonstrate that this is caused by Mn<sup>2+</sup>-dependent binding of PsaR to and Zn<sup>2+</sup>-dependent release from the promoters of these genes. Thus, these data represent an intriguing insight in the opposite regulatory effects of two metal cations on the expression of a set of virulence genes, mediated by a single transcriptional repressor.

The concentrations as well as the ratio of Mn<sup>2+</sup> and Zn<sup>2+</sup> may vary greatly between different sites in the human body. For example, in lung tissue the total concentration of Mn<sup>2+</sup> is approximately 0.2 μg/g (wet weight) (3.6 μM) and that of Zn<sup>2+</sup> is 15 μg/g (wet weight) (229 μM), whereas in the blood serum concentrations of Mn<sup>2+</sup> and Zn<sup>2+</sup> are 0.5 ng/ml (9 nM) and 1.0

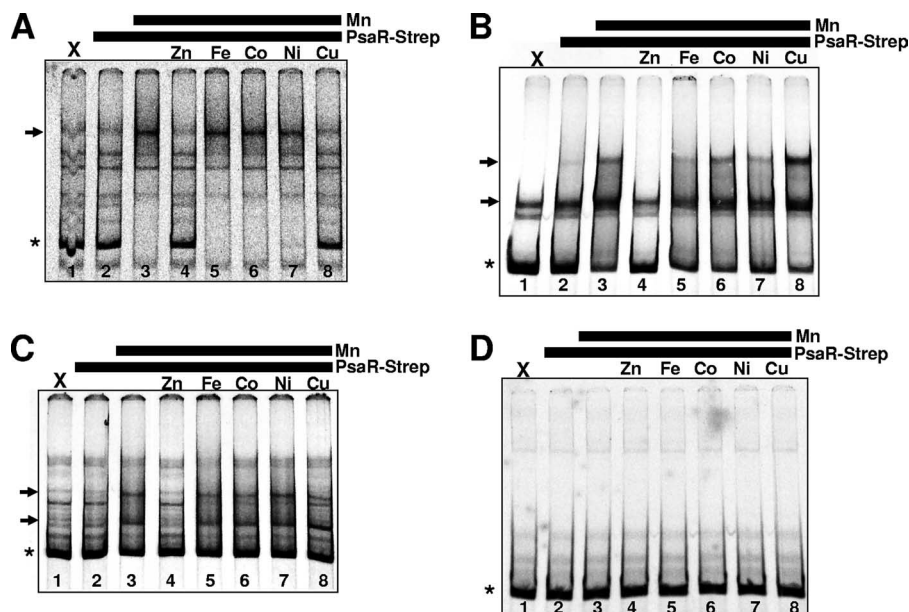


FIG. 3. In vitro interaction of Psar-Strep with the *pcpA* (A), *psaB* (B), and *prtA* (C) promoter regions and with a truncated *PcpA* fragment lacking the Psar operator (D). Purified Psar-Strep was added at concentrations of 5 nM (*PcpA*), 11 nM (*PprtA*), and 25 nM (*PpsaB*). Metal ions were added as indicated above the lanes at a concentration of 50  $\mu$ M. X, free probe. The horizontal bar above lanes 3 to 8 indicates the presence of  $Mn^{2+}$ , and the horizontal bar above lanes 2 to 8 indicates the presence of Psar-Strep. Arrows indicate the positions of the shifted probes, and asterisks indicate the position of the free probe. The presence of weaker bands which run higher than the free probe in the gels is a phenomenon that has also been seen by others in similar experiments. These bands may represent unspecific PCR products or single-stranded DNA (1, 15).

$\mu$ g/ml (15.3  $\mu$ M), respectively (81). Control of availability of both ions is of importance to the host, as a recent study showed that the human immune system employs chelation of  $Mn^{2+}$  and  $Zn^{2+}$  by calprotectin as a way to inhibit bacterial growth in tissue abscesses (13). On the other hand, sufficiently high levels of  $Zn^{2+}$  are required for proper functioning of the immune system (72). Thus, it is likely that the concentrations of  $Mn^{2+}$  and  $Zn^{2+}$  fluctuate greatly in the environment, which will lead to varying concentrations of these metal ions in the cytoplasm of *S. pneumoniae*. However, virulence studies that have been carried out so far with respect to *pcpA*, *prtA*, and *psaBCA* do not point to a specific site where the proteins encoded by these genes are needed (7, 26, 31, 47, 50, 51, 62). Interestingly, *pcpA* is also regulated by the nutritional regulator CodY (26), meaning that the concentrations of both metal ions as well as amino acids affect *pcpA* expression.

Homologs of Psar in other organisms seem to have slightly different functions. In *Streptococcus gordonii*, ScaR is an  $Mn^{2+}$ -dependent repressor of the Sca ( $Mn^{2+}$ ) permease (29). In *Streptococcus pyogenes*, MtsR regulates the  $Mn^{2+}$ -specific ABC transporter MtsABC in response to  $Mn^{2+}$ , while the heme-specific HtsABC transporter is repressed by MtsR in response to  $Fe^{3+}$  (23). The *Streptococcus mutans* SloR regulates several genes involved in biofilm formation, genetic competence, oxidative stress tolerance, and adherence in response to  $Mn^{2+}$  and, to a lesser extent,  $Fe^{3+}$  (35, 63, 67, 74).

The EMSAs performed in this study are in line with the transcriptional data and suggest that a direct effect of  $Zn^{2+}$  and  $Mn^{2+}$  on the Psar-promoter interaction causes the observed transcriptional effects. However, in the EMSAs  $Mn^{2+}$  does not overcome the  $Zn^{2+}$  effect at equimolar concentration, whereas in vivo, only low (but repressive) concentrations of

$Mn^{2+}$  are counteracted by  $Zn^{2+}$ . This might be because of different intracellular concentrations/availabilities of these metal ions compared to the extracellular concentrations and indicates that in vivo  $Mn^{2+}$  is the principal effector. It will be interesting to know why  $Zn^{2+}$  and  $Mn^{2+}$  have these opposite effects on the DNA binding properties and activity of Psar.

Clues about this could come from recent structural studies on DtxR from *Corynebacterium diphtheriae* (14) and MntR, a DtxR family protein from *Bacillus subtilis* that responds to  $Mn^{2+}$  (65). Both DtxR and MntR contain two metal binding sites per monomer: a low-affinity site and a high-affinity site (14, 21). MntR binds metal ions with affinities that roughly follow the Irving-Williams series, where  $Mn^{2+}$  displays the lowest affinity for MntR and  $Zn^{2+}$  the highest (21). As MntR has a much higher affinity for  $Zn^{2+}$  than  $Mn^{2+}$  but only very poorly activates DNA binding of MntR, the specificity of MntR is not correlated with the metal binding affinity (21). The conformation of  $Mn^{2+}$ -bound MntR differs from the  $Zn^{2+}$ -bound conformation with respect to the occupancy of the metal binding sites:  $Mn^{2+}$  binds to two sites, whereas only one  $Zn^{2+}$  ion binds to MntR, which does not allow binding of a second one (36). Metal binding at the second site is proposed to be required for DNA binding, as it promotes a disorder-to-order transition of MntR structure (17). Psar shares 25% and 15% sequence identity with DtxR and MntR, respectively. Moreover, sequence alignment shows that most residues that constitute the metal ion binding sites in DtxR and MntR are conserved in Psar (data not shown). Therefore, it might be that  $Zn^{2+}$  prevents  $Mn^{2+}$  binding to Psar, rendering Psar in a monomeric or destabilized state, and in this way counteracts  $Mn^{2+}$ -induced DNA binding and transcriptional repression.

Interference with the effect of one metal ion on a metal-

sensory protein by another metal ion has been reported recently for CzcA in *B. subtilis* (24). CzcA normally is activated for DNA release in the presence of  $Zn^{2+}$ , but  $Cu^{2+}$  inhibits the  $Zn^{2+}$ -induced allostery, since in vitro the protein preferentially binds  $Cu^{2+}$ . However, these effects are not seen in vivo. High levels of  $Cu^{2+}$  in the growth medium induces the Fur regulon in *B. subtilis* (56). Thus, opposite effects of metals on regulation of gene expression seems to occur with other classes of metalloregulatory proteins as well.

The identified PsaR binding site is similar to operator sequences of PsaR homologs in other streptococcal species. *S. gordonii* ScaR binds to a similar region in the *scaA* promoter but also to a second inverted repeat (29). This second repeat is present in the promoter region of *psaB* in *S. pneumoniae* but not in the promoters of *pcpA* and *prtA*. SloR in *S. mutans* also likely exerts its repressive effect on *sloABC* through a larger palindromic sequence that includes the conserved region that we identify (35). Apart from the promoters of *prtA*, *pcpA*, and *psaBCA*, no others that contain the identified PsaR operator in their promoter regions could be found in the R6 and TIGR4 genomes. This suggests that the PsaR regulon consists of only these genes in *S. pneumoniae*, in contrast to the case for *S. mutans*, where SloR directly regulates a large number of genes (67). It is very likely that also in TIGR4 the activity of PsaR is dependent on both  $Zn^{2+}$  and  $Mn^{2+}$ , since, apart from one amino acid difference (Asn161→Ser), TIGR4 PsaR is identical to the R6 and D39 PsaR sequences (data not shown). Johnston et al. (31) also found a repressive effect of  $Mn^{2+}$  and PsaR on the expression of the *rlrA* pathogenicity islet (SP0461 to SP0465). The *rlrA* locus is not present in the genomes of D39 and R6, making the effect of PsaR on *rlrA* and the downstream genes a strain-dependent phenomenon. There is a possible PsaR operator in the *rlrA* promoter region with a perfectly conserved first half-site, but a very degenerate second half-site, 5'-AAATTAAACAACCTTCCATC-3' (consensus bases are in bold). Point mutations in the conserved bases of the first half of the operator destroyed PsaR-dependent regulation for the *pcpA* promoter (Table 8). However, we did not test the effect of mutations in the second half-site. Therefore, it cannot be excluded that an operator consisting of an intact first repeat and a degenerate inverted repeat, as is the case for the putative operator in the *rlrA* promoter, is still able to confer some weak PsaR-dependent regulation. As RlrA activates expression of the genes downstream of *rlrA*, namely, *rrgA*, *rrgB*, *rrgC*, and *srtB* (25), weak repression of *rlrA* by PsaR likely explains the up-regulation of the *rlrA* locus in the *psaR* mutant (31, 68).

In conclusion, this study indicates that the relative availabilities of  $Zn^{2+}$  and  $Mn^{2+}$  in the human body could modulate the expression of several virulence genes and in this way affect the outcome of infection by *S. pneumoniae*.

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